eISSN 2255-0569

ORIGINAL

Effects of sex hormones disruption, after prenatal and postnatal exposure to chlordimeform, on monoaminergic neurotransmitters systems in female and male rat's prefrontal cortex

Efectos de la disrupción de las hormonas sexuales, tras la exposición prenatal y posnatal al Clordimeformo, sobre los sistemas de neurotransmisores monoaminérgicos en la corteza prefrontal de ratas macho y hembra

José Manuel García[1], Paula Moyano[1], María Teresa Frejo[2], María José Anadón[1], Miguel Andrés Capó[2], Gloria Gómez[1], Javier del Pino[2]

1. Departamento de Toxicología y Legislación Sanitaria, Facultad de Medicina, Universidad Complutense of Madrid, Spain.
2. Departamento de Toxicología y Farmacología, Facultad de Veterinaria, Universidad Complutense of Madrid, Spain.

Correspondencia
Javier del Pino PhD
Facultad de Veterinaria. Universidad Complutense de Madrid
Departamento de Toxicología y Farmacología
Avda. Puerta de Hierro s/n · 28040, Madrid
Teléfono: 913 550 920
E-mail: jdelpino@pdi.ucm.es

Recibido: 21 – XI – 2016
Aceptado: 22 – XII – 2016

doi: 10.3306/MEDICINABALEAR.32.01.11

Abstract

Introduction: Chlordimeform, as well as other formamidine pesticides, has been described to induce permanent sex- and region-dependent effects on development of monoaminergic neurotransmitter systems. The mechanisms that induce these effects are not known, but it has been suggested that these effects could be related to monoamine oxidase (MAO) inhibition. However, chlordimeform is a very weak MAO inhibitor, which suggest that other mechanism should be involved. In this regard, formamidines, in general, and chlordimeform, in particular, alter the serum levels of steroid hormones, which regulate the expression of enzymes that mediate the synthesis and metabolism of monoaminergic neurotransmitters systems. Therefore, an alteration of these hormones in the brain could mediate the effects observed.

Objectives and methods: In order to confirm that the formamidines produce permanent alterations of the monoamine neurotransmitter systems, through disruption of sex hormones in the brain, by alteration of the expression of the enzymes that synthesize and/or metabolize these neurotransmitters, we evaluated, in frontal cortex of male and female rats, the effect on the levels of testosterone and estradiol at 11 days of age, as well as the expression of MAO, COMT, BDH, TH, TRH, and AD enzymes at 60 days of age after maternal exposure to chlordimeform (5 mg/kg body weight).

Results: Chlordimeform induced a significant decrease in testosterone and estradiol levels in frontal cortex of rats at 11 days of age. We observed sex interaction with treatment in the content of T and E2. We determined a bigger increase in the expression of TH [35,66% (P<0,001)] and TRH [42,14% (P<0,001)] enzymes in males than in females. Chlordimeform treatment did not alter the expression of MAO, COMT, AD, BDH enzymes, but decreased the expression of the enzymes TRH TH in both males and females.

Conclusions: The present findings indicate that after maternal exposure to formamidines, in general, and chlordimeform, in particular, a permanent alteration of monoaminergic neurotransmitters, through alteration of the enzymes that synthetize these neurotransmitters, mediated by sex hormones disruption in frontal cortex is induced.

Keywords: Chlordimeform; formamidines; neurodevelopmental toxicity; TH; TRH; rats; human risk assessment

Resumen

Introducción: Se ha descrito que el clordimeformo, así como otros plaguicidas formamidínicos, induce alteraciones permanentes de los sistemas de neurotransmisores monoaminérgicos región y sexo dependiente. Los mecanismos por los que se inducen estos efectos no se conocen, pero se ha sugerido que podrían estar relacionados con la inhibición de la monoamino oxidasa (MAO). Sin embargo, el clordimeformo es un inhibidor muy débil de la MAO lo que sugiere que otro mecanismo debería estar implicado. En este sentido, se ha descrito que las formamidinas en general y el clordimeformo en particular, alteran los niéveles séricos de distintas hormonas las cuales regulan la expresión de las enzimas que sintetizan y metabolizan estos neurotransmisores. Por lo tanto, una alteración de estas hormonas a nivel cerebral podría mediar los efectos observados.

Objetivos y métodos: Con el objetivo de confirmar que las formamidinas produce alteraciones permanentes de los neurotransmisores monoaminérgicos, a través de la disrupción de las hormonas sexuales a nivel cerebral por alteración de la expresión de las enzimas que sintetizan y/o metabolizan estos neurotransmisores, se evaluaron los efectos, en la corteza frontal de ratas macho y hembra, sobre los niveles de testosterona y estradiol a los 11 días de edad, así como sobre la expresión de las enzimas MAO, COMT, BDH, TH , TRH, y AD a los 60 días de edad tras la exposición maternal al clordimeformo (5 mg/kg de peso corporal).

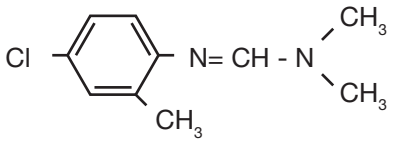
Resultados: El clordimeformo indujo una disminución significativa de los niveles de testosterona y estradiol en la corteza frontal de las ratas descendientes a la edad de 11 días. Se observó una interacción por sexo con el tratamiento en el contenido de T y E2. Además se observó una mayor expresión de las enzimas TH [35,66% (P<0,001)] y TRH [42,14% (P<0,001)] en los machos que en las hembras. El tratamiento con clodimeformo no alteró la expresión de las enzimas MAO, COMT, AD, BDH, pero disminuyó la expresión de las enzimas TH y TRH tanto en machos como en hembras.
Conclusiones: Los presentes resultados indican que las formamidinas, en general, y el clordimeformo, en particular, inducen, tras la exposición maternal, una alteración permanente de los sistemas de neurotransmisores monoaminergicos en la corteza frontal, a través de la alteración de las enzimas que sintetizan estos neurotransmisores, mediada por la alteración de las hormonas sexuales.

Palabras clave: Clordimeformo; formamidinas; neurotoxicidad en el desarrollo; testosterona; estradiol; TH, TRH, ratas; evaluación del riesgo para el hombre

Introduction

Formamidine pesticides have been reported to induce permanent alteration of brain development. In this regard, the formamidine compound amitraz has been described to produce the induction of permanent alterations on the development of central nervous system (CNS) such as those that affect monoamine neurotransmitter systems[1]. Moreover, chlordimeform [N2-(4-chloro-o-tolyl)-N1. N1-dimethylformamidine] (**Figure 1**), which is another member of formamidines family, has also been reported to induce permanent alterations of serotoninergic, noradrenergic and dopaminergic systems[2,3]. The mechanism by which these effects occur is not known.

Figure 1: Chlodimeform chemical structure (C10H13Cl N2).



Currently, it is assumed that the monoaminergic neurotransmitters play a role during development, defined as "morphogenetic" [4-7]. Any change in the levels of catecholamines during development could have a profound effect on brain development, both structural and functional[8]. In this sense, monoamine oxidase (MAO) inhibition was among the first biochemical actions of the formamidines to be reported[9-10] and has been suggested as a possible mechanism of action, because neuronal MAO participates in metabolic inactivation of biogenic monoamines, which include the neurotransmitters serotonin (5-HT), norepinephrine (NE), and dopamine (DA). However, chlordimeform is a very weak MAO inhibitor, which suggest other mechanisms are involved in these effects.

Alternatively, the changes in NE, DA and 5-HT and its metabolites levels observed in rats' brain after formamidines exposure could be attributed to a possible effect on sex steroid hormones that modulate the expression of enzymes such as tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH), tryptophan hydroxylase (TRH), MAO, catechol-O-metyltransferase (COMT), aldehyde dehydrogenase (AD), aldehyde reductase (AR) required for synthesis and metabolism of these neurotransmitters[11-18]. In this regard, chlordimeform is an endocrine disruptor that alters prolactin and adrenocorticotropic hormones, among others[19], and amitraz alters testosterone and estradiol serum hormone levels in rats[20]. Therefore, if chordimeform, as well as other formamidine pesticides, alters sex hormones in the brains, this could mediate the effect observed on monoaminergic neurotransmitters systems.

According to all exposed above, we performed a study to establish if maternal exposure to formamidines during gestation and lactation induces permanent alterations on the enzymes that synthetize and metabolize 5-HT, NE and DA neurotransmitters in adult age, through sex hormones disruption. Chlordimeform was chosen because it is the most representative compound in its group, which presents a very low inhibition of MAO, allowing us to study more clearly whether the permanent changes observed on levels of these neurotransmitters are due to an alteration of the enzymes that catalyze the synthesis and metabolism of these neurotransmitter rather than inhibition of MAO.

This work focuses its interest in providing new data of formamidines induced neurotoxicity during nervous system development, because new compounds of this family are being developed with therapeutic applications for which these effects are not considered in their risk assessment, which poses a potential health hazard.

Material and methods

Biological material

All experiments were performed in accordance with European Union guidelines (2003/65/CE) and Spanish regulations (BOE 67/8509-12, 1988) regarding the use of laboratory animals. Eight pregnant Wistar rats were housed individually in polycarbonate cages and were assigned randomly to two experimental groups: a chlordimeform treatment group (n = 4) and a control group (n = 4).

Test Chemical and Treatment

Chlordimeform (Sigma, Madrid, Spain) was dissolved in

corn oil to provide fast and complete absorption and was administered orally by gavage in a volume of 2 mg/ml. The animals received daily chlordimeform at the dose of 5 mg/kg on days 6 to 21 of pregnancy (GD 6-21) and on days 1 to 10 of lactation (PN 1-10). Control dams received vehicle (corn oil 2.5 ml/kg) on the same schedules. Dose of chlordimeform was selected based on a previous preliminary study that indicated that this dose was the higher one that did not cause weight loss or mortality, reduction of food or water intake as well as did not induce haematological modifications of other clinical histopathological signs of overt toxicity. None of the prenatal or postnatal treatment evoked a significant change in weight of any of the brain regions on PN 60 (data not shown).

Dams were examined daily throughout the gestation and lactation periods for mortality, general appearance and behaviour. The maternal body weights were measured on GD 1, GD 5, GD 6, GD 15 and GD 20. Food and water consumption during pregnancy, length of gestation, litter size and sex ratio were also assessed.

On PN1, all litters were examined externally, sexed and weighed. Litters were organized in groups of twenty-four pups, twelve males and twelve females. Litters were weighed at PN 1, PN 7, PN14 and PN 21. The offspring were weaned on lactation day 21 and were maintained in appropriate conditions, housed individually and without any treatment with full access to food and water until adult age. The study was organized in treated groups of six males and six females randomly selected respectively from the dams' litters exposed to chlordimeform, and control groups of six males and six female's pups randomly selected respectively from the control dams' litters.

At PN11, for the analysis of testosterone and estradiol's brain levels and at PN 60, for the analysis of MAO A, MAO B, COMT, BDH, AD, TH and TRH gene expression, male and female rats from control and treated groups (pups from control dams, and pups from dams exposed to chlordimeform, respectively) were sacrificed by decapitation. The brain was removed quickly and the frontal cortex was a rapidly dissected out at 4ºC[21], since this brain region was previously describe to present sex differences in the effect observed on these neurotransmitters systems and to be one of the most affected[2,3]. Tissues were rapidly weighed and stored at -80ºC until analysis. All data were collected by experimenters blind to the treatment condition of the offspring.

Estradiol and testosterone quantification

Estradiol and testosterone content were measured in prefrontal cortex from treated animals in order to determine whether sex hormones are altered by chlordimeform exposure. Estradiol and testosterone content in the prefrontal cortex was measured using an enzyme immunoassay kit (Estradiol EIA Kit, Cayman Chemical Company, MI, USA), according to the manufacturer's instruction. Tissues were homogenized in 300 µl of an equal mixture of ethyl acetate and 0.1 M phosphate-buffered saline. The homogenates were centrifuged at 21,000 g for 15 min at 4ºC. The resulting mixture was then incubated in a MeOH/dry ice bath to solidify the aqueous phase (bottom) and the organic phase was eluted into a new tube. The ethyl acetate portion was collected and dried. The dried material was reconstituted in 120 µl EIA buffer, and 100 µl of the sample was used for EIA at duplicate. ELISA values were obtained (pg/ml) and corrected for weigh tissue (mg/ml), producing a final unit of pg/mg and presented as a percentage of the untreated control.

Real-time PCR analysis

The MAOA, MAOB, COMT, AD, TH, TRH and DBH expression was measured in frontal cortex tissue from control and chlordimeform treated animals in order to determine whether chlordimeform, through sex hormones disruption, alters permanently the expression of these enzymes. Total RNA was extracted using the Trizol Reagent method (Invitrogen, Madrid, Spain). The final RNA concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain), and the quality of total RNA samples was assessed using an Experion LabChip (Bio-Rad, Madrid, Spain) gel. First-strand cDNA was synthesized with 1000 ng of cRNA by using a PCR array first strand-synthesis kit (C-02; SuperArray Bioscience, Madrid, Spain) in accordance with the manufacturer's instructions and including a genomic DNA elimination step and external RNA controls. After reverse transcription, QPCR was carried out using prevalidated primer sets (SuperArray Bioscience) for mRNAs encoding MAOA (PPR46359A), COMT (PPR06789A), AD (PPPR43520B), TH (PPR45220F), TRH (PPR48244A), DBH (PPR52652A), and ACTB (PPM02945B). ACTB was used as an internal control for normalization. Reactions were run on a CFX96 using Real-Time SYBR Green PCR master mix PA-012 (SuperArray Bioscience). The thermocycler parameters were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 72°C for 30 seconds. Relative changes in gene expression were calculated using the Ct (cycle threshold) method. The expression data are presented as actual change multiples[22].

Data analysis

Statistical analysis of data was performed using a Statgraphics software, version Plus 4.1 for windows. Values are expressed as mean ± S.E.M. obtained from 12 animals, six males and six females, in each group (control and treated groups). For values combined for males and females, a two-way ANOVA with treatment × sex interaction was the initial test used. Where a significant treatment × sex interaction was detected, a separate Student's t test was carried out for each sex. The results were considered significant at $P<0.05$. Results significantly different from controls are also presented as change from control (%).

Results

Maternal and offspring body weight, physical and general activity development were unaffected by the exposure of dams to chlordimeform (5 mg/kg bw orally on days 6 to 21 of pregnancy and 1 to 10 of lactation).

Estradiol and testosterone quantification

Oral treatment with chlordimeform to dams during the gestation period from day 6 to day 21 and during lactation from day 1 to day 10 affected the content of T and E2 in the region of frontal cortex of rats offspring at the age of 11 days. The content of T (ng/g tissue) in the region of frontal cortex in the control group and treated group is presented in **table I**. The content of E2 (ng/g tissue) in the region of the frontal cortex of the control group and the treated group is presented in **table II**. The results expressed in **tables I** and **II** show that in 11 days old rats treated during gestation days 6-21 and during lactation days 1-10 through their mothers, a statistically significant loss of E2 and T content in the frontal cortex compared to control animals was produced. A sex interaction with treatment in the content of T and E2 was observed (**Figure 2**). In frontal cortex the loss observed of E2 content was 46.53% (P <0.01) and 57.39% (P <0.001) in males and females, respectively, and the loss in the content of T was 13.44% (P <0.001) and 20.70% (P <0.001) in males and females, respectively (**Figure 2**).

Real-time PCR analysis

Oral treatment with chlordimeform to dams during the gestation period from day 6 to day 21 and during lactation from day 1 to day 10 affected the TH and TRH gene expression of rats offspring at the age of 60 days. In 60 days old rats treated during gestation days 6-21 and during lactation days 1 to 10 a decrease in the expression of TH and TRH enzymes in frontal cortex with respect to control animals was observed. No effect on gene expression of MAO, COMT, BDH and AD enzymes was observed (**Figure 3**). A sex difference in TH and THR gen expression was observed, being higher the expression of TH [35,66% (P<0,001)] and THR [42,14% (P<0,001)] in males than females rats (**Figure 4**).

Table I: Tissue T (pg/ml) content determined in frontal cortex from male and female rats at 11 days of age treated with vehicle or chlordimeform (5 mg/kg bw orally on days 6 to 21 of pregnancy and 1 to 10 of lactation).

	Frontal Cortex			
Animal	**Control group Males**	**Treated group Males**	**Control group Females**	**Treated group Females**
1	397,56	344,56	403,76	310,56
2	401,45	348,62	395,91	321,65
3	385,87	341,67	387,76	306,75
4	396,74	338,59	389,56	311,39
5	399,50	331,99	408,75	318,95
6	388,71	345,81	401,64	323,84
Mean ± SEM	**394,97±2,54*** **	**341,87±2,43*** (-13,44%)**	**397,90±3,38*** **	**315,52±2,81*** (-20,70)**

Values are mean ± S.E.M.; control animals (n= 6 males, n= 6 females); treated group (n= 6 males, n= 6 females).
Statistical significance is reported for the ****P*<0.001 levels compared with the control group.

Table II: Tissue E2 (pg/ml) content determined in frontal cortex from male and female rats at 11 days of age treated with vehicle or chlordimeform (5 mg/kg bw orally on days 6 to 21 of pregnancy and 1 to 10 of lactation).

	Frontal Cortex			
Animal	**Control group Males**	**Treated group Males**	**Control group Females**	**Treated group Females**
1	132,56	73,98	128,65	55,44
2	133,65	69,44	125,74	51,76
3	136,59	64,32	131,78	48,87
4	129,54	75,93	129,75	60,76
5	128,65	72,59	134,87	57,53
6	131,72	67,62	126,64	56,89
Mean ± SEM	**132,12±1,17*** **	**70,65±1,76*** (-46,53%)**	**129,57±1,38*** **	**55,21 ±1,74*** (-57,39%)**

Values are mean ± S.E.M.; control animals (n= 6 males, n= 6 females); treated group (n= 6 males, n= 6 females).
Statistical significance is reported for the ****P*<0.001 levels compared with the control group.

Figure 2: Tissue T and E2 (pg/ml) content determined in frontal cortex from male and female rats at 11 days of age treated with vehicle or chlordimeform (5 mg/kg bw orally on days 6 to 21 of pregnancy and 1 to 10 of lactation).

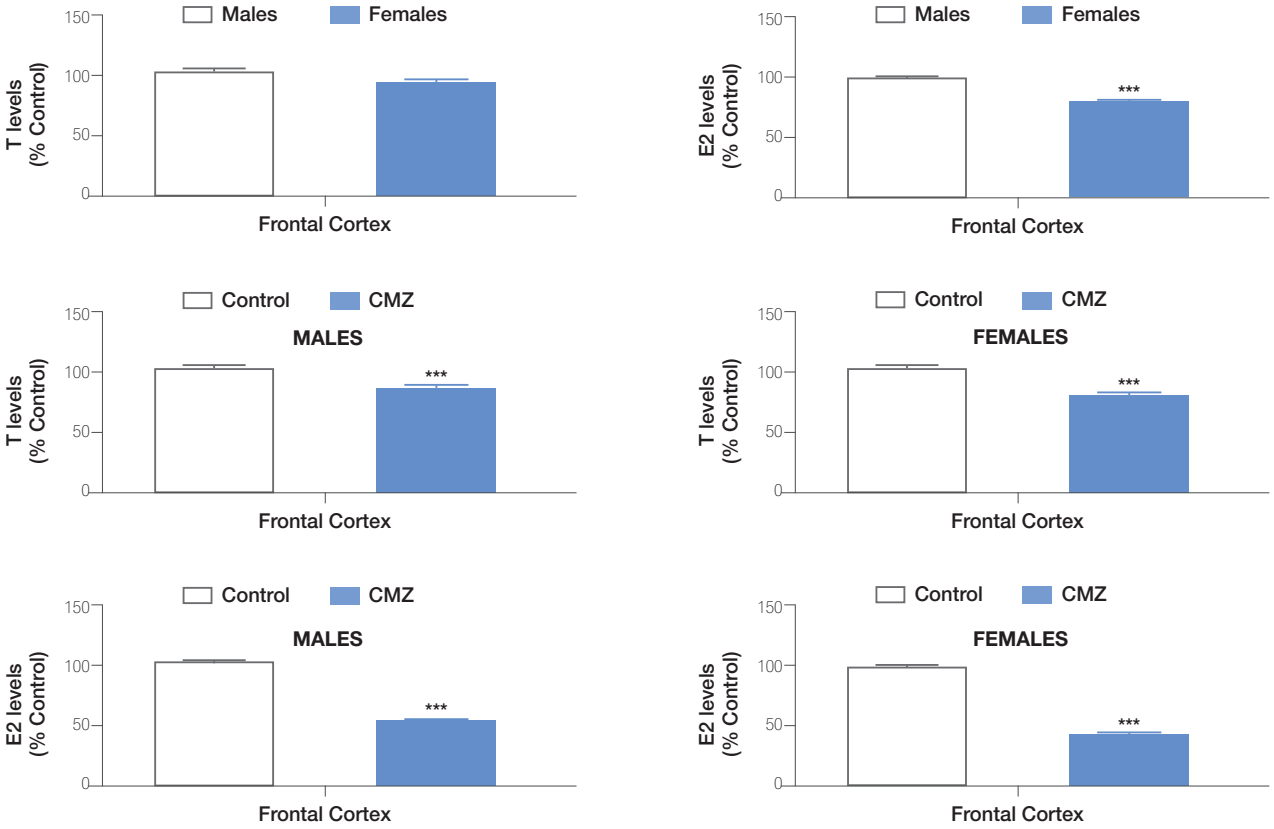


Figure 3: Sex difference results from real-time PCR targeting MAO, COMT, BDH, AD, TH y TRH genes after chlordimeform treatment in male and female rats. MAO, COMT, BDH, AD, TH y TRH gene expression was compared to male rats results. Each bar represents mean ± SD of 6 samples. Levels were measured using QPCR. ACTB was used as an internal control. ***p ≤ 0.001,**p ≤ 0.01, significantly different from males.

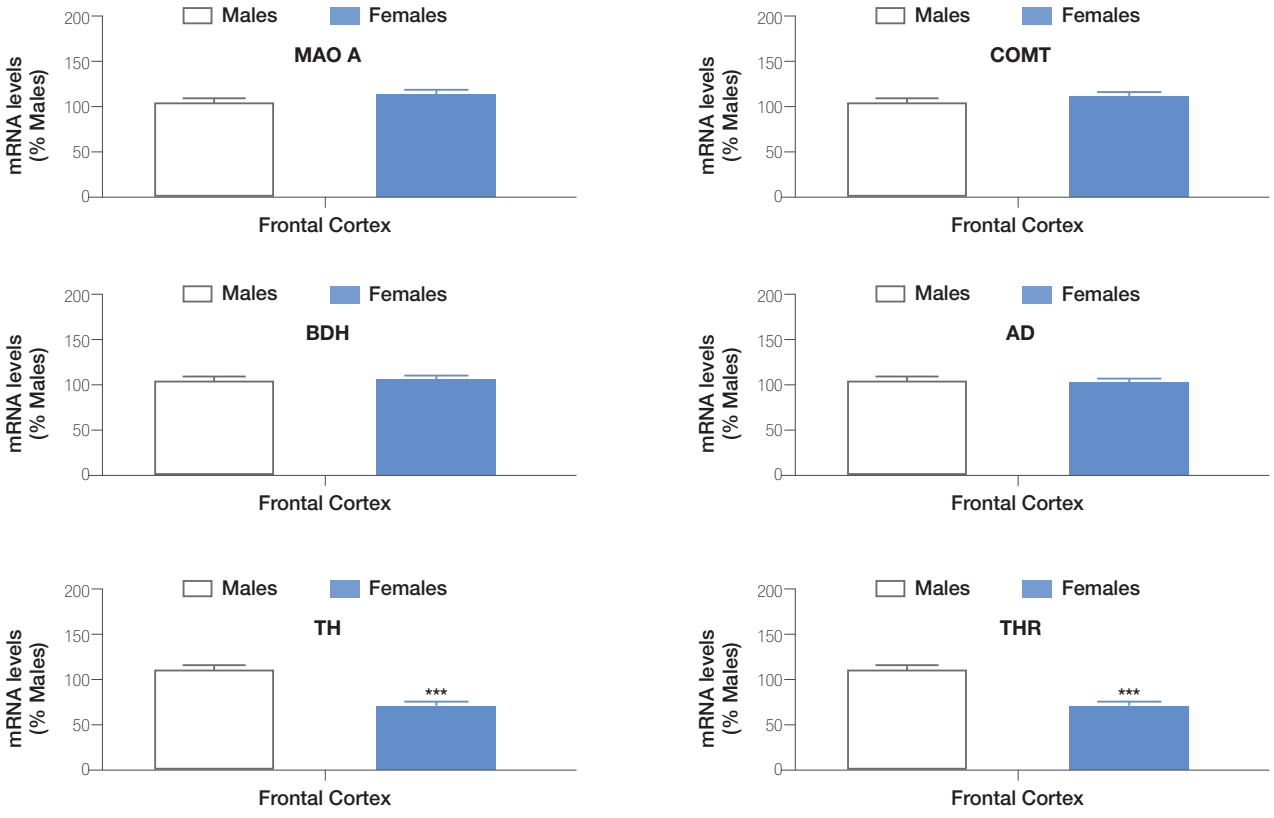
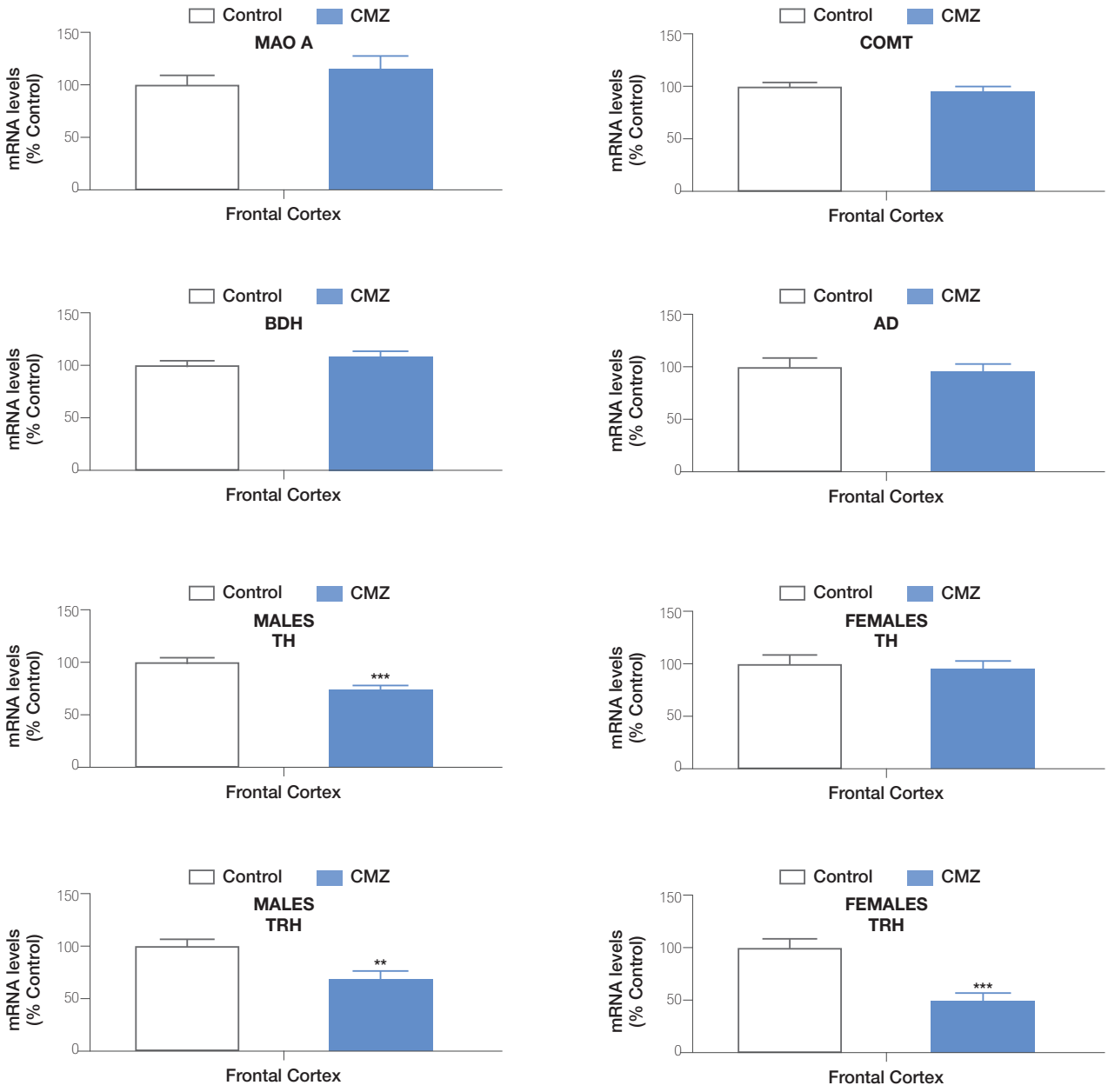


Figure 4: Results from real-time PCR targeting MAO, COMT, BDH, AD, TH y TRH genes after chlordimeform treatment in male and female rats. MAO, COMT, BDH, AD, TH y TRH gene expression was compared to controls. Each bar represents mean ± SD of 6 samples. Levels were measured using QPCR. ACTB was used as an internal control. ***p ≤ 0.001, **p ≤ 0.01 significantly different from controls.



Discussion

Developmental neurotoxicity involves alterations in behavior, neurohistology, neurochemistry and/or gross dysmorphology of CNS occurring in the offspring, because of chemical exposure of dams during pregnancy or lactation. Previous studies described that formamidines induce permanent alteration in developing monoamine neurotransmitter systems[1-3]. The mechanism by which these permanent effects on monoaminergic systems take place is unknown, but monoamine neurotransmitters regulate brain development prior to assuming their roles as transmitters in the mature brain[23-25], thus any circumstance that affects these neurotransmitters in the developing brain can alter the final structure and function of that brain. Since the endogenous levels of 5-HT, DA and NE are highly regulated by MAO, any change in this enzyme can profoundly affect the developing brain. In this regard, it has been reported that gestational exposure to MAO inhibitors clorgyline and deprenyl produc-

es in offspring at 30 days of age, a significant reduction of serotonergic innervation particularly in the frontal cortex[26], but not in the dopaminergic and noradrenergic innervation, which suggests that besides MAO inhibition other mechanism should be implicated in the alteration observed. However, chlordimeform is a very week MAO inhibitor[27-29], but presents similar permanent regional and sexual dependent effects than amitraz, which is a potent MAO inhibitor[9]. These data suggest that MAO inhibition could not produce the alterations in monoaminergic neurotransmitters systems observed, confirming that other mechanisms are involved.

Otherwise, steroids play a role in the development of catecholamine systems[30-33], and play a critical role in mammalian brain developmental of both genders[34]. The present study shows that prenatal and postnatal exposure to chlordimeform (5 mg/kg bw orally on days 6 to 21 of pregnancy and 1 to 10 of lactation) was not able to induce maternal toxicity, since during pregnancy maternal weight gain of treated rats was not modified. However, chlordimeform administered during pregnancy and lactation leads to a decrease in T and E2 levels at PN11, which is the critical period of time when sexual differentiation takes place, in males and females rats' frontal cortex. This effect produced a permanent reduction of the TH and TRH gene expression, which catalyse the synthesis of monoaminergic neurotransmitters, at 60 days of age in males and females rats' frontal cortex. Previously, chlordimeform has been reported to disrupt different steroids hormones[19], which support the effect observed. The origin of the sex hormones in the brain, could be from gonads or from endogenous synthesis, as previously described, whose contribution to the final effect depends on the region and sex steroid hormone[35-38]. Estradiol alters the levels of enzymes that synthesize DA, NE and 5HT, as well as those that degrade these neurotransmitters[12,14,16,39-40]. E2 elevated mRNA levels of TH, the first and major rate limiting enzyme in catecholamine biosynthesis[40] and enhanced TRH mRNA expression[12]. In addition, T and DHT regulated the synthesis and metabolism of monoamines[17]. In this sense, T and DHT increased TH protein and COMT, MAO-A and MAO-B mRNAs[15]. In the same way, DHT decreased neurotransmitter turnover of DOPAC/DA, MHPG/NE, and 5-HIAA/5-HT of gonadectomized animals[13]. These previous data support the effects observed on these enzymes after chlordimeform treatment, and so, on the monoaminergic neurotransmitters.

Furthermore, other possible mechanisms that may contribute to the permanent alterations observed on monoaminergic neurotransmitters systems could be a direct action of chlordimeform on neuronal cell replication, differentiation, axonogenesis and synaptogenesis and functional development of neurotransmitter systems, effects that could result in behavioural alterations observed in previous studies after developmental exposure to chlordimeform[41]. The loss of dopaminergic, serotoninergic and noradrenergic projections could also play an important role in the behavioural alterations. In this regard, frontal cortex participates in the regulation of learning and memory processes[42-45], thus, it could be considered that these processes could be compromised by exposure during gestation and lactation to formamidines. In addition, the dysfunction in serotonin and dopamine systems are involved in appetite, affective, neuropsychiatric disorders[46-49], among others, which could also be induced by formamidine exposure during development. Further studies are needed to test whether these other mechanisms described could be involved in the effects observed and to confirm that alteration of these neurotransmitter systems is the cause of some of these dysfunctions.

Given that the DA, 5-HT and NE systems alterations observed after chlordimeform exposure in the frontal cortex, striatum, and hippocampus was similar between them[2,3], and was also the same as those affected by amitraz[1], it could be inferred that the alteration in the expression of these enzymes, mediated through sex hormones disruption, is the mechanism by which these monoaminergic neurotransmitters are altered in these brain regions by chlordimeform and formamidines in general. Further studies are needed to confirm whether this mechanism and others, probably involved in these effects, are the same in all brain regions studied and for all formamidines.

Conclusion

In summary, our results suggest that the mechanism by which the alterations in the development of the monoaminergic neurotransmitter systems in frontal cortex is mediated through disruption of estradiol and testosterone levels, which produced a permanent alteration of the expression of some of the enzymes that synthetize and metabolize these monoaminergic neurotransmitters. Further studies are required to check whether other hormones are also involved in these effects and to determine whether they act directly on expression of the affected enzymes or through induction of other genes that can regulate their expression. Otherwise, it should be determined whether there is a reduction in innervation in the regions affected that could also contribute to the effect observed. Due to the fact that monoaminergic neurotransmitters dysfunctions are related with appetite, affective, neurological and psychiatric disorders, behavioral studies of formamidines are also needed to clarify the outcomes of long-term alterations in these monoaminergic neurotransmitters systems. Currently, new molecules with therapeutic application are being developed as N-hydroxy-N-(4-butyl-2-methylphenyl) formamidine (HET0016) with protective effects against cardiovascular and cerebrovascular diseases. Until now, the risk assessment of the family of these compounds

has been taken from the standpoint of carcinogenesis. In view of these results and our previous results it might be appropriate to reconsider the risk assessment of the members of this family based not only on their possible carcinogenic effects, but also in the neurotoxic effects during development mediated by endocrine disruption. The results reported in this study are of great importance and should be incorporated into the risk assessment of pesticides formamidines group.

Compliance with ethical standards

All experiments were performed in accordance with European Union guidelines (2003/65/CE) and Spanish regulations (BOE 67/8509-12, 1988) regarding the use of laboratory animals.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors would like to thank Miguel Capo and Margarita Lobo, Professors of Toxicology from the Universidad Complutense de Madrid for their counseling during the preparation of the present work.

References

1. Del Pino J, Martínez MA, Castellano VJ, Ramos E, Martínez-larrañaga MR, Anadón A. Effects of prenatal and postnatal exposure to amitraz on norepinephrine, serotonin and dopamine levels in brain regions of male and female rats. Toxicology. 2011; 287(1-3):145-52.

2. García JM, Alias P, Frejo MT, Anadon MJ, Capo MA, Del Pino J. Effects of prenatal and postnatal exposure to chlordimeform on serotonin levels in brain regions of adult's male and female rats. Medicina Balear. 2015; 30(1): 21-6

3. García JM, Frejo MT, Anadon MJ, Capo MA, Del Pino J. Permanent sexual and regional noradrenergic and dopaminergic systems impairment after prenatal and postnatal exposure to chlordimeform. 2015; 30(3): 12-8.

4. Buznikov GA, Shmukler YB, Lauder JM. From oocyte to neuron: do neurotransmitters function in the same way throughout development. Cell. Mol. Neurobiol. 1996; 16(5): 533-59.

5. Levitt P, Harvey JA, Friedman E, Simansky K, Murphy EH. New evidence for neurotransmitter influences on brain development. Trends Neurosci. 1997; 20(6):269-74.

6. Nicotra A, Schatten G. Propranolol, a beta-adrenergic receptor blocker, affects microfilament organization, but not microtubules, during the first division in sea urchin eggs. Cell Motil. Cytoskeleton. 1990; 16(3):182-9.

7. Nicotra A, Senatori O. Some characteristics of mitochondrial monoamine oxidase activity in eggs of carp (Cyprinus carpio) and rainbow trout (Salmo gairdneri). Comp. Biochem. Physiol. 1989; C92(2):401-4.

8. Lakshmana M, Raju TR. Endosulfan induces small but significant changes in the levels of noradrenaline, dopamine and serotonin in the developing rat brain and deficits in the operant learning performance. Toxicology. 1994; 91(2):139-50.

9. Aziz SA, Knowles CO. Inhibition of monoamine oxidase by the pesticide chlordimeform and related compounds. Nature. 1973; 242:417-8.

10. Bailey BA, Martin RJ, Downer RGH. Monoamine oxidase inhibition and brain catecholamine levels in the rat following treatment with chlordimeform. Pest Biochem Physiol. 1982; 17:293-300.

11. De Souza Silva MA, Mattern C, Topic B, Buddenberg TE, Huston JP. Dopaminergic and serotonergic activity in neostriatum and nucleus accumbens enhanced by intranasal administration of testosterone. Eur Neuropsychopharmacol. 2009; 19 (1): 53–63.

12. Donner N, Handa RJ. Estrogen receptor-beta regulates the expression of tryptophan hydroxylase 2 mRNA within serotonergic neurons of the rat dorsal raphe nuclei. Neuroscience. 209; 163: 705–18.

13. Handa RJ, Hejna GM, Lorens SA. Androgen inhibits neurotransmitter turnover in the medial prefrontal cortex of the rat following exposure to a novel environment. Brain Res. 1997; 751 (1): 131–8.

14. Luine VN, Rhodes JC. Gonadal hormone regulation of MAO and other enzymes in hypothalamic areas. Neuroendocrinology. 1983; 36: 235–41.

15. Purves-Tyson TD, Handelsman DJ, Double KL, Owens SJ, Bustamante S, Weickert CS. Testosterone regulation of sex steroid-related mRNAs and dopamine-related mRNAs in adolescent male rat substantia nigra. BMC Neurosci1. 2012; 3: 95.

16. Scardapane L, Cardinali DP. Effect of estradiol and testosterone on catecholmethyl transferase activity of rat superior cervical ganglion, pineal gland, anterior hypophysis and hypothalamus. J Neurotrans. 1977; 40: 81–6.

17. Thiblin I, Finn A, Ross SB, Stenfors C. Increased dopaminergic and 5-hydroxytryptaminergic activities in male rat brain following long-term treatment with anabolic androgenic steroids. Br J Pharmacol. 1999; 126 (6): 1301–6.

18. Lubbers LS, Zafian PT, Gautreaux C, Gordon M, Alves SE, Correa L, Lorrain DS, Hickey GJ, Luine V. Estrogen receptor (ER) subtype agonists alter monoamine levels in the female rat brain. J Steroid Biochem Mol Biol. 2010; 122 (5): 310–7.

19. Stoker TE, Goldman JM, Cooper RL, McElroy WK. Influence of chlordimeform on alpha-adrenergic receptor-associated mechanisms of hormonal regulation in the rat: pituitary and adrenocortical secretion. Toxicology. 1991; 69:257-68.

20. Chou CP, Lu SY, Ueng TH. Modulation of serum concentrations and hepatic metabolism of 17 -estradiol and testosterone by amitraz in rats. Arch Toxicol. 2008; 82 (10): 729–37.

21. Glowinski J, Iversen LL. Regional studies of catecholamines in the rat brain-I. The disposition of [3H] norepinephrine, [3H] dopamine and [3H]DOPA in various regions of the brain. J. Neurochem. 1966; 13:655-69.

22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25: 402-8.

23. Whitaker-Azmitia PM. Role of serotonin and other neurotransmitter receptors in brain development: basis for developmental pharmacology. Pharm. Rev. 1992; 43:553-61.

24. Di Pino G, Moessner R, Lesch KP, Lauder JM, Persico AM. Roles for serotonin in neurodevelopment: more than just neural transmission. Curr. Neuropharmacol. 2004; 2:403-17.

25. Ansorge MS, Morelli E, Gingrich JA. Inhibition of serotonin but not norepinephrine transport during development produces delayed, persistent perturbations of emotional behaviours in mice. J. Neurosci. 2008; 28(1):199-207.

26. Whitaker-Azmitia PM, Zhang X, Clarke C. Effects of gestational exposure to monoamine oxidase inhibitors in rats: preliminary behavioral and neurochemical studies. Neuropsychopharmacology. 1994; 11(2):125-32.

27. Neumann R, Voss G. MAO inhibition, an unlikely mode of action for chlordimeform. Experientia. 1977; 33: 23-4.

28. Robinson CP, Smith PW. Lack of involvement of monoamine oxidase inhibition in the lethality of acute poisoning by chlordimeform. J Toxicol Environ Health. 1977; 3(3): 565-8.

29. Hollingworth RM. Chemistry, biological activity, and uses of formamidine pesticides. Environ Health Perspect. 1976; 14: 57-69.

30. Stewart J, Rajabi H. Estradiol derived from testosterone in prenatal life affects the development of catecholamine systems in the frontal cortex in the male rat. Brain Res. 1994; 646(1):157-60.

31. Leret ML, Rua C, Garcia-Montojo M, Lecumberri M, González JC. Influence of metyrapone treatment during pregnancy on the development and maturation of brain monoaminergic systems in the rat. Acta Physiol (Oxf). 2009; 197(4):333-40.

32. Muneoka K, Kuwagata M, Ogawa T, Shioda S. Sex-specific effects of early neonatal progesterone treatment on dopamine and serotonin metabolism in rat striatum and frontal cortex. Life Sci. 2010; 87(23-26):738-42.

33. Pappas SS, Tiernan CT, Behrouz B, Jordan CL, Breedlove SM, Goudreau JL, Lookingland KJ. Neonatal androgen-dependent sex differences in lumbar spinal cord dopamine concentrations and the number of A11 diencephalospinal dopamine neurons. J Comp. Neurol. 2010; 518(13): 2423-36.

34. Konkle ATM, McCarthy MM. Developmental time course of estradiol, testosterone, and dihydrotestosterone levels in discrete regions of male and female rat brain. Neuroendocrinology. 2011; 152(1): 223-35.

35. Hojo Y, Higo S, Ishii H, Ooishi Y, Mukai H, Murakami G, Kominami T, Kimoto T, Honma S, Poirier D, Kawato S. Comparison between hippocampus-synthesized and circulation-derived sex steroids in the hippocampus. Endocrinology. 2009; 150(11): 5106-12.

36. Robel P, Bourreau E, Corpéchot C, Dang DC, Halberg F, Clarke C, Haug M, Schlegel ML, Synguelakis M, Vourch C. Neuro-steroids: 3 beta-hydroxy-delta 5-derivatives in rat and monkey brain. J Steroid Bioche. 1987; 27(4-6): 649-55.

37. Zwain IH, Yen SS. Dehydroepiandrosterone: biosynthesis and metabolism in the brain. Endocrinology. 1999; 140(2):880-7.

38. Zwain IH, Yen SS. Neurosteroidogenesis in astrocytes, oligodendrocytes, and neurons of cerebral cortex of rat brain. Endocrinology.1999; 140(8): 3843-52.

39. Luine VN, Khylchevskaya RI, McEwen BS. Effect of gonadal steroids on activities of monoamine oxidase and choline acetylase in rat brain. Brain Res. 1973; 86: 293–306.

40. Serova L, Rivkin M, Nakashima A, Sabban EL. Estradiol stimulates gene expression of norepinephrine biosynthetic enzymes in rat locus coeruleus. Neuroendocrinology. 2002; 75: 193–200.

41. Olson KL, Boush GM, Matsumura F. Behavioral effects of perinatal exposure of chlodimeform in rats. Bull Environ Contam Toxicol. 1978; 20(6):760-8.

42. Cifariello A, Pompili A, Gasbarri A. 5-HT (7) receptors in the modulation of cognitive processes. Behav Brain Res. 2007; 195(1):171-9.

43. González-Burgos I, Feria-Velasco A. Serotonin/dopamine interaction in memory formation. Prog Brain Res. 2008; 172:603-23.

44. Lapiz MD, Morilak DA. Noradrenergic modulation of cognitive function in rat medial prefrontal cortex as measured by attentional set shifting capability. Neuroscience. 2006; 137: 1039–49.

45. Dunnet SB, Meldrum A, Muir JL. Frontal-striatal disconnection disrupts cognitive performance of the frontal-type in the rat. Neuroscience. 2005; 135:1055–65.

46. Insel TR, Zohar J, Benkelf ATC, Murphy DL. Serotonin in obsessions, compulsions, and the control of aggressive impulses. Ann. N. Y. Acad. Sci. 1990; 600:574-85.

47. Stein DJ, Hollander E, Liebowitz MR. Neurobiology of impulsivity and the impulse control disorders. J. Neuropsychiatry Clin. Neurosci. 1993; 5(1):9-17.

48. Riederer P, Sofic E, Konradi C, Kornhuber J, Beckmann H, Dietl M, Moll G, Herbenstreit G. The role of dopamine in control of neurobiological functions. In: Fluckiger E, Muller EE, Thorner MO. (Eds.), Basic and Clinical Aspects of Neuroscience. The Role of Brain Dopamine. 1989. Springer. Berlin, pp. 1-17.

49. Kaye W. Neurobiology of anorexia and bulimia nervosa. Physiol Behav. 2008; 94:121-35.